

Parturition invokes changes in peripheral blood mononuclear cell populations in Holstein dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Johne's disease (JD) is characterized by a protracted period of subclinical infection. Infected cows may remain in the subclinical state until stressors such as parturition and lactation invoke more clinical signs of disease. The objective of this study was to evaluate changes in the percentages of CD4⁺, CD8⁺, and $\gamma\delta$ T-cells, B-cells, monocytes, as well as the expression of the activation marker, CD5, on these cell subpopulations in the peripheral blood of dairy cows naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) during the periparturient period. Peripheral blood mononuclear cells (PBMCs) were collected from 3 wk pre- to 4 wk post-calving and freshly isolated or cultured for 7 d. Day 7 cultures were infected with live MAP at a 10:1 MOI (bacteria to adherent PBMC), and cultures were incubated for an additional 24 h. Fluorescent antibody labeling of lymphocyte subsets and monocytes was conducted and analyzed with flow cytometry. Freshly isolated PBMCs from subclinical cows expressed a greater ($P < 0.05$) percentage of CD8⁺ and $\gamma\delta$ T-cells compared with clinical cows. The percentage of CD4⁺ T-cells increased ($P < 0.08$) in clinical cows as parturition approached. During the postpartum period, clinical cows had greater ($P < 0.05$) CD4:CD8 ratios compared with subclinical and control cows. After 8 d, uninfected PBMCs from clinical cows had greater ($P < 0.05$) percentages of CD14⁺ cells compared with subclinical cows. When infected with live MAP, there was no effect of infection group or parturition on cell subpopulations. In fresh PBMCs, clinical cows expressed lower percentages of CD4⁺CD5^{bright} and CD8⁺CD5^{bright} compared with control cows, but greater percentages of CD5^{dim} cells for all lymphocyte subsets. These results suggest changes in the percentages of lymphocyte subsets, monocytes, and CD5 markers are modulated by both infection status and the periparturient period.

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1. Introduction

Johne's disease (JD), caused by the intracellular pathogen *Mycobacterium avium* subsp. *paratuberculosis*

(MAP), is estimated to infect more than 22% of US dairy herds and cost the US dairy industry up to \$250 million annually (Ott et al., 1999). In general, dairy cows will become infected with MAP as neonates through fecal–oral transmission. Once infected, cows may remain in the subclinical, or asymptomatic, stage of the disease for several years (Larsen et al., 1975). Stressors, such as parturition, may induce the transition from subclinical to clinical stage of the disease. Clinical

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animals are characterized by fecal shedding of the bacteria, intermittent but persistent diarrhea, progressive weight loss, and eventual death. The exact mechanism that triggers the progression of the disease remains unknown.

On-farm observations suggest that dairy cows infected with MAP may demonstrate increased signs of clinical disease during the weeks following parturition. Research on what prompts the progression of disease from the asymptomatic subclinical state to a more clinical state during this time period is lacking. The periparturient period, defined as 3 wk prior to and the 3 wk following parturition, represents a time of physiological stress for the dairy cow. Metabolic changes such as the rapid increase in non-esterified fatty acid concentrations (Radcliff et al., 2003; Karcher et al., 2007), accompanied by a postpartum decline in both blood glucose (Radcliff et al., 2003) and calcium concentrations (Kimura et al., 2006), present challenges to the dairy cow. Rapid fluctuations in both serum progesterone and estradiol during the periparturient period contribute additional stress (Weber et al., 2001; Radcliff et al., 2003). In addition, the cow is forced to deal with immunosuppression characterized by decreased lymphocyte function (Kehrli et al., 1989a; Meglia et al., 2005) and the decreased ability of neutrophils to migrate and phagocytize (Kehrli et al., 1989b; Lee and Kehrli, 1998). Parturition has a major impact on the number of T- and B-cells, both components of the adaptive immune system, and the number of monocyte/macrophages, effectors of the innate immune system, in the peripheral blood of healthy dairy cows. Studies have noted dramatic decreases in the percentage of peripheral blood CD4⁺ T-cells and $\gamma\delta$ T-cells at parturition (Van Kampen and Mallard, 1997; Kimura et al., 1999). In contrast, increased activity of CD8⁺ lymphocytes has been observed in cows at calving compared with mid to late lactating cows (Shafer-Weaver and Sordillo, 1997). The percentage of B-cells in peripheral blood was highest immediately prior to and lowest immediately following parturition (Van Kampen and Mallard, 1997). In addition, the number of monocytes and monocyte-derived macrophages were increased at calving (Kimura et al., 2002). The total number and percentages of CD4⁺ and CD8⁺ (α/β T-cells), $\gamma\delta$ T-cells, and B-cells, plays a significant role in the ability of the animal to respond to an infection.

In paratuberculosis, the progression from a subclinical to a clinical stage of disease is characterized by a shift from cell-mediated (Th1) immunity to an antibody-mediated (Th2) humoral response. This shift

in Th1 to Th2 immunity is characterized by a decreased percentage of peripheral blood T-cells and an increase in the percentage of B-cells for clinically infected cows (Waters et al., 1999; Koets et al., 2002). More specifically, the percentages of $\gamma\delta$ and CD4⁺ T-cells in peripheral blood are remarkably decreased in clinical cows compared with healthy controls (Koets et al., 2002). The CD4:CD8 ratio is also decreased in chronically infected animals as the number of CD8⁺ T-cells does not seem overtly affected by the transition to a clinical disease state (Koets et al., 2002). The decline in CD4⁺ T-cells observed in clinical cows further illustrates the compromised nature of the immune system as these cells are key effectors of Th1-mediated immunity through the secretion of IFN- γ , a cytokine that is credited for controlling mycobacterial infections (Cooper et al., 1993; Flynn et al., 1993). Immunosuppressive T regulatory cells (CD4⁺CD25⁺) are upregulated in the ileum of MAP-infected dairy cows (Weiss et al., 2006) and have been shown to secrete an abundance of IL-10 and reduced IFN- γ compared with CD4⁺CD25⁻ (Belkaid et al., 2002).

To date, limited research is available characterizing detailed aspects of periparturient immunosuppression in the dairy cow. Further, it is not clear what impact the periparturient period and its associated stressors may have on host immunity in cows with paratuberculosis. Therefore, the objective of this study was to determine the percentages of CD4⁺, CD8⁺, and $\gamma\delta$ T-cells, B-cells, and monocytes in the peripheral blood of dairy cows naturally infected with MAP during the periparturient period as compared with healthy control cows. In addition, cell populations were further delineated by staining for CD5, a marker for T- and B-cell activation.

2. Materials and methods

2.1. Animals

Twenty-one multiparous Holstein cows and two primiparous Holstein cows (age range from 3 yr to 6 yr) were grouped according to infection status. These groups consisted of (1) noninfected healthy control cows ($n = 5$), (2) cows naturally infected with MAP, but asymptomatic ($n = 14$) and (3) naturally infected cows with clinical Johne's disease ($n = 4$). The two primiparous cows were both in the subclinically infected group. The stage of infection was determined by fecal shedding of MAP and IFN- γ . Infection was monitored bacteriologically for the fecal shedding of MAP by standard culture methods (Stabel, 1997). By definition,

clinical animals were shedding more than 100 CFU/tube of media and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/tube of media. The noninfected control cows were characterized by repeated negative fecal cultures performed quarterly over a 3–5-yr period and had been purchased from herds with no recent history of Johne's disease. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for MAP and IFN- γ) performed during that period. Whereas subclinically infected cows had high antigen-specific IFN- γ responses and negligible Ab titers and clinically infected cows had low to moderate antigen-specific IFN- γ responses and high Ab titers. All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA).

2.2. Blood collection and culture conditions

Blood was collected from the jugular vein in 2× acid-citrate–dextrose (ACD; 1:10). For each animal, blood was collected at –21, –14, –7, +1, +7, +14, +21, and +28 d relative to parturition. Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat fractions of blood. PBMCs were resuspended in complete media [RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Atlanta Biologics, Atlanta, GA), 100 U of penicillin G sodium (Gibco, Grand Island, NY) per mL, 100 μ g of streptomycin sulfate (Gibco, Grand Island, NY) per mL, 0.25 μ g of amphotericin B (Gibco, Grand Island, NY) per mL, and 2 mM L-glutamine (Gibco, Grand Island, NY)]. In 96-well round bottom plates (Corning Incorporated, Corning, NY), 50 μ L of the cell suspension was added to wells containing 50 μ L of primary monoclonal antibody to CD4⁺, CD8⁺, $\gamma\delta$ T-cells, B-cells, monocytes, and CD5 (Table 1). All wells received 10 μ g/mL

of DAPI (4'-6-diamidino-2-phenylindole; Sigma, St. Louis, MO) to differentiate live from dead cells and allow gating on viable cells. Cells were then incubated at 4 °C for 30 min. After incubation, plates were centrifuged at 400 × g for 2 min at 4 °C and the supernatant decanted. Secondary antibody cocktail (100 μ L) consisting of fluorescein-conjugated anti-mouse IgM (Southern Biotech, Birmingham, AL), R-phycoerythrin-conjugated goat F(ab)2 anti-mouse IgG2a (Southern Biotech, Birmingham, AL), and peridinin-chlorophyll-protein complex-conjugated rat anti-mouse IgG1 (Becton Dickinson, San Jose, CA) diluted 1:312, 1:625, and 1:42, respectively, in PBS with 1% fetal calf serum and 0.04% sodium azide was then added to designated wells and the plate was centrifuged again at 400 × g for 2 min at 4 °C. The cells were then suspended in 200 μ L of BD FACS Lyse (BD Biosciences, San Jose, CA) for immediate flow cytometric analysis.

Additional cells were cultured in complete medium at 2.0×10^6 /mL in 48-well flat-bottomed plates (Corning Incorporated, Corning, NY). Cells were incubated for 7 d at 39 °C in 5% CO₂ in a humidified atmosphere and then evaluated using flow cytometric analyses. Replicate wells were set up concurrently and incubated under the same conditions for enumeration of monocyte-derived macrophages in the cultures. For counting purposes, nonadherent cells were removed from the plates after 7 d of incubation and the wells were washed with cold 1× PBS to remove the adherent cells from the plates. The adherent cells were approximately 80–90% monocyte-derived macrophages as determined by staining and were quantified with a cell counter prior to the addition of live MAP. *M. avium* subsp. *paratuberculosis* strain K-10 (NADC) was then added at a ratio of 10 bacteria per adherent PBMC to the experimental wells containing the total PBMC population (Khalifeh and Stabel, 2004). Control wells (medium alone; 8 d control PBMC) and infected wells (MAP infected; 8 d infected PBMC) were then

Table 1
Primary antibodies^a

Antigen	MAb clone	Isotype	Working MAb concentration (μ g/mL) ^b	Specificity
CD4	GC50A1	IgM	14	T-Helper cell
CD5	B29a	IgG _{2a}	7	Activation marker
CD8	BAQ111A	IgM	14	T-cytotoxic/suppressor cell
CD14	CAM36A	IgG ₁	14	Monocytes/macrophages
N12	CACT61A	IgM	14	$\gamma\delta$ -Cell receptor
B lymphocyte	BAQ155A	IgG ₁	7	Total B-cell

^a VMRD Inc. (Pullman, WA).

^b Diluted in PBS with 1% fetal calf serum and 0.04% sodium azide.

incubated for an additional 24 h. Cells then were prepared for flow cytometric analysis by using the same procedure as described above.

2.3. Bacteria

M. avium subsp. *paratuberculosis* strain K-10 (NADC) was prepared as previously described (Khalifeh and Stabel, 2004). Bacteria were grown in Middlebrook 7H9 broth (pH 6.0) supplemented with mycobactin J (2 mg/L; Allied Monitor, Fayette, MO) and oleic acid–albumin–dextrose complex (Becton Dickinson Microbiology, San Jose, CA). The bacteria were harvested, washed 3× with PBS (pH 7.4; 0.15 M), and resuspended in PBS to a final concentration of 10^9 /mL as determined by the absorbance at 540 nm. Bacterial stocks then were frozen in PBS at -80°C until used in the experiments. Prior to in vitro infection, frozen bacterial stocks were thawed, and clumps were dispersed by brief sonication at 25 W for 40 s with a Tekmar sonic disturber (Tekmar, Lorton, VA). The viable cells in the frozen bacterial stocks were determined by culturing on HEYM. Viable cells in stocks typically were decreased after thawing and sonication to approximately 10^8 CFU/mL.

2.4. Flow cytometric analysis

Samples were evaluated by analyzing 30,000 events per sample using a FACScan flow cytometer (Cell Quest Software; Becton Dickinson, San Jose, CA). Analysis was conducted by gating on mononuclear cells based on forward and side scatter characteristics (FlowJo, Tree Star, Inc., Ashland, OR). The percentage of CD4^+ , CD8^+ , $\gamma\delta$ T-cells, B-cells, and monocyte/macrophage populations were determined from the gated mononuclear cell population. Percentages of CD5^+ cells within these cell populations also were determined.

2.5. Statistical analysis

Percentages of each cell population were analyzed by using the PROC mixed analysis of SAS (PROC MIXED in SAS[®] PC Windows Version 9.1.3 software). For repeated measurements, the model included the fixed effects of infection group and days relative to parturition, random effects of cow within infection group by time, the interactions of fixed effects, and the residual error. The covariance structure utilized was compound symmetry in all analyzed parameters. Significant differences because of treatment group

were considered different if $P < 0.05$ and considered a tendency towards significance if $0.05 \leq P \leq 0.15$. Values were reported as least square means \pm S.E. of the mean unless noted otherwise.

3. Results

3.1. Mononuclear cell populations in freshly isolated PBMCs

An overall effect of parturition ($P < 0.05$) caused a significant decline in the percentage of total mononuclear cells from -7 d to $+1$ d relative to parturition for all animals regardless of infection status (data not shown). Values returned to pre-calving values by $+14$ d. On $+7$ d and $+21$ d after parturition, clinical and subclinical cows had decreased ($P < 0.05$) numbers of mononuclear cells compared with the control cows. A significant ($P < 0.05$) effect of parturition was observed throughout the periparturient period for the percentage of CD4^+ cells in freshly isolated PBMC (Fig. 1A). Compared with the prepartum period, the percentage of CD4^+ T-cells increased at parturition for clinically infected cows ($P < 0.08$), whereas healthy control cows showed a gradual decline in number of CD4^+ T-cells from -21 d to -7 d. A highly significant ($P < 0.01$) interaction of infection group and parturition was observed throughout the study. At $+7$ d and $+21$ d, subclinically and clinically infected cows had a greater ($P < 0.01$) percentage of CD4^+ T-cells in freshly isolated total PBMCs compared with the control cows. There was an overall effect of infection group on the percentage of CD8^+ T-cells in fresh PBMCs (Fig. 1B). On average, the percentage of CD8^+ T-cells was greater ($P < 0.05$) in subclinically infected cows ($20.3\% \pm 2.1$) compared with clinically infected ($10.3\% \pm 3.77$) and control ($10.3\% \pm 3.3$) cows. An interaction of infection group and parturition was observed ($P < 0.01$) as subclinical cows had greater percentages of CD8^+ T-cells compared with the other groups on all sampling days during the postpartum period.

The $\text{CD4}:\text{CD8}$ was dissimilar within the infected groups with higher ($P < 0.01$) $\text{CD4}:\text{CD8}$ ratios noted for clinical compared to subclinical cows (Table 2). There was a significant ($P < 0.05$) infection group and parturition interaction with higher $\text{CD4}:\text{CD8}$ ratios observed on d $+1$, $+7$, and $+14$ of the study.

In fresh PBMCs, the overall percent of $\gamma\delta\text{-TCR}^+$ cells was lower ($P < 0.01$) in clinical cows ($1.7\% \pm 0.9$) compared with subclinical cows ($4.4\% \pm 0.5$), with a tendency ($P < 0.06$) to be lower than control cows

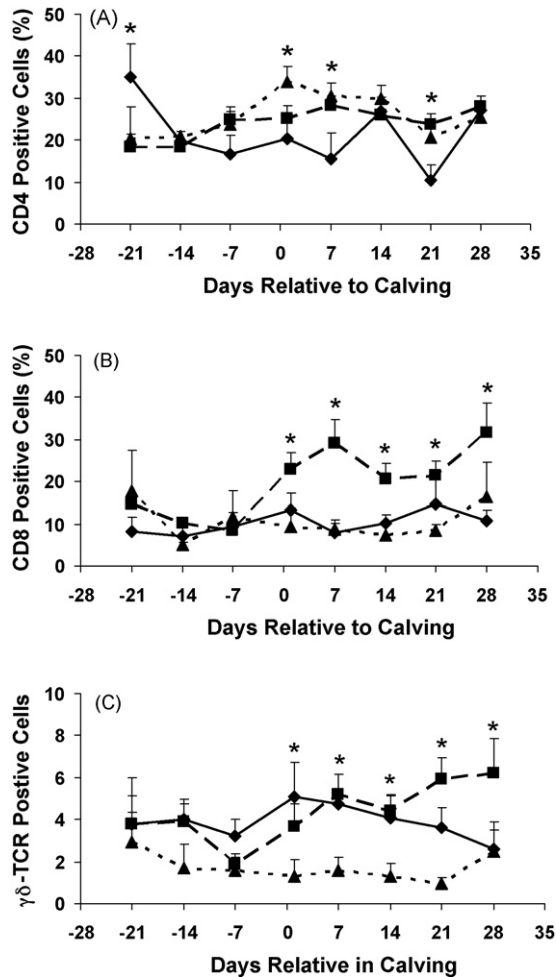


Fig. 1. Percentage of positive mononuclear cells from fresh PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) CD4⁺ T-cells. There was an effect of parturition ($P < 0.05$) and an interaction of infection group and parturition ($P < 0.01$). (B) CD8⁺ T-cells. There was an effect of parturition and interaction of infection group and parturition ($P < 0.01$). (C) $\gamma\delta$ T-cells. Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

($3.9\% \pm 0.8$) (Fig. 1C). There was no significant effect of parturition; however, both control and subclinical cows tended to have increased ($P < 0.12$) numbers of $\gamma\delta$ T-cells as parturition approached, with further increases noted for subclinically infected cows in the postpartum period.

There was no effect of infection group or parturition on the percentage of B-cells in this study (Fig. 2A). However, the percentage of B-cells from subclinical cows declined as parturition approached ($P < 0.03$). An increase in the percentage of B-cells from clinical cows was noted from -14 d to $+7$ d ($P < 0.06$). Similarly, there was no effect of infection or parturition on the percentage of CD14⁺ cells in fresh PBMCs (Fig. 2B), but a trend for an interaction of infection group and parturition ($P < 0.12$) was observed. Subclinical cows expressed a greater ($P < 0.05$) percentage of CD14⁺ cells on $+7$, $+14$, and $+21$ d compared with control and clinical cows.

3.2. Effects of in vitro infection with *M. avium* subsp. *paratuberculosis* on mononuclear cell populations in 8 d cultures

PBMCs from clinically infected cows incubated with medium alone had an overall lower ($P < 0.05$) percentage of total mononuclear cells ($61.3\% \pm 3.2$) compared with both control ($71.2\% \pm 2.8$) and subclinically infected cows ($68.5\% \pm 1.6$) (data not shown). Differences because of infection status were only observed during the prepartum period (infection group \times parturition effect, $P < 0.01$). For control and subclinically infected cows, the percentage of mononuclear cells declined from -7 d to $+1$ d and rapidly increased by $+7$ d (data not shown). Similar trends were observed after in vitro infection with MAP, with lower percentages of mononuclear cells noted for clinically infected cows during the prepartum period compared with control and subclinical cows (infection group \times parturition effect, $P < 0.10$) (Fig. 3). A significant

Table 2

CD4:CD8 ratios across the periparturient period for fresh peripheral blood mononuclear cells isolated from healthy control, subclinical and clinical cows naturally infected with MAP

Infection status	Days relative to calving							
	-21	-14	-7	+1	+7	+14	+21	+28
Control	4.03 \pm 0.7 a	3.32 \pm 1.1	2.41 \pm 1.0	2.03 \pm 0.6 a	2.58 \pm 1.0 a	2.21 \pm 0.2 a	1.11 \pm 0.6	2.34 \pm 0.4
Subclinical	1.93 \pm 0.4 b	2.18 \pm 0.4	2.42 \pm 0.5	1.78 \pm 0.4 a	1.61 \pm 0.6 a	1.22 \pm 0.2 a	1.52 \pm 0.4	1.45 \pm 0.4
Clinical	1.97 \pm 1.8 ab	3.43 \pm 1.4	3.20 \pm 1.3	6.08 \pm 1.3 b	4.78 \pm 1.5 b	5.49 \pm 0.1 b	2.98 \pm 0.9	2.52 \pm 1.1

Letters indicate differences between infection groups within a given time point ($P < 0.05$). Infection group, $P < 0.03$; Parturition, $P < 0.11$; Infection group \times parturition, $P < 0.05$.

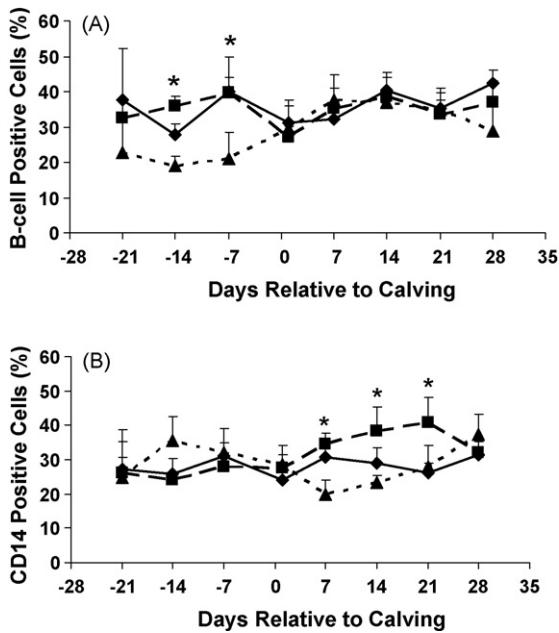


Fig. 2. Percentage of positive mononuclear cells from fresh PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) B-cells and (B) CD14⁺ cells. Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

($P < 0.05$) overall effect of parturition was observed for clinical cows as the number of mononuclear cells increased from -7 d to $+7$ d and gradually decreased again by $+28$ d. Although not statistically significant, the control and subclinical cows had a decline in mononuclear cells from -14 d to $+1$ d (Fig. 3). There was no overall effect of infection group on the percentage of CD4⁺ T-cells from 8 d control PBMCs (Fig. 4A). A trend for an effect of parturition ($P < 0.09$) was noted with both control and clinical cows

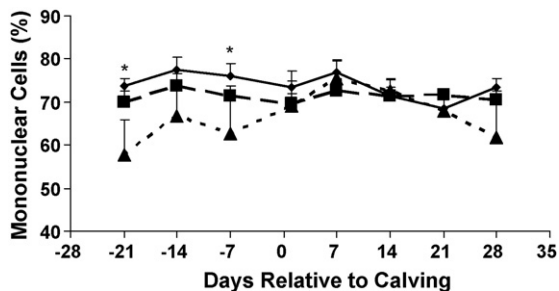


Fig. 3. Percentage of mononuclear cells from 8 d infected PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. There was an effect of parturition for clinical cows ($P < 0.05$). Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

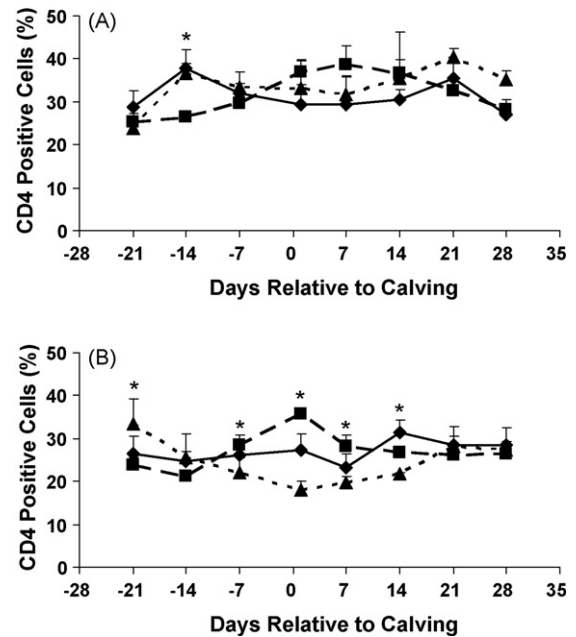


Fig. 4. Percentage of positive CD4⁺ T-cells from 8 d PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) Control cells and (B) infected cells. There was an interaction between infection group and parturition ($P < 0.01$). Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

demonstrating a decline in CD4⁺ T-cells as parturition approached. By $+21$ d, the percent had returned to prepartum values. Subclinical cows were unique in that they showed a 32% increase in CD4⁺ T-cells beginning at -14 d and continuing until $+7$ d (Fig. 4A). By $+28$ d, the percentage had returned to -14 d prepartum values. For 8 d infected PBMC cultures, there was a significant interaction of infection group and parturition ($P < 0.01$) (Fig. 4B). For subclinical cows, the percentage of CD4⁺ T-cells increased from $21.2\% \pm 2.1$ at -14 d to $35.6\% \pm 2.2$ at $+1$ d ($P < 0.01$), followed by a decline during the postpartum period. Interestingly, in contrast to subclinically infected cows, the percentage of CD4⁺ T-cells decreased from $33.4\% \pm 5.8$ at -21 d to $18.0\% \pm 2.2$ at $+1$ d for clinically infected cows, rebounding to $28.1\% \pm 4.6$ by $+21$ d. The percentage of CD4⁺ T-cells from control animals remained relatively constant during the periparturient period until an increase was noted at $+14$ d.

There was no overall effect of infection group on the percent of CD8⁺ T-cells from 8 d control PBMC; however, an increase in CD8⁺ T-cells was observed at parturition for all cows (Fig. 5). Interestingly, clinical cows had a brief increase ($P < 0.01$) in CD8⁺ T-cells from $15.7\% \pm 0.7$ at $+7$ d to $31.6\% \pm 5.1$ at $+14$ d. No effect of parturition or infection group was observed on

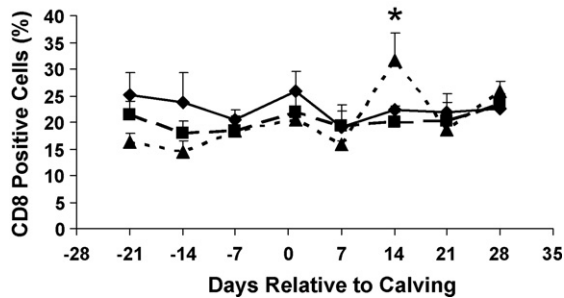


Fig. 5. Percentage of positive CD8 T-cells from 8 d control PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. There was an effect of parturition ($P < 0.05$). Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

the CD8⁺ population in 8 d infected PBMCs (data not shown). There was no effect of infection group on the CD4:CD8 ratios in either control or infected 8 d PBMC; however, a significant decrease ($P < 0.01$) in CD4:CD8 was observed for control and clinically infected cows as parturition approached (data not shown).

There was no overall effect of infection group on the percentage of $\gamma\delta$ -TCR⁺ or B-cells from control or 8 d infected PBMCs (data not shown). However, a significant ($P < 0.04$) effect of parturition was noted on +1 d with clinical cows having higher numbers of B-cells in 8 d control PBMC ($70.8\% \pm 7.6$) compared with subclinical ($56.7\% \pm 4.7$) and control ($45.8\% \pm 4.6$). Clinical cows had a greater ($P < 0.05$) percentage of CD14⁺ cells in 8 d control PBMC ($39.1\% \pm 2.7$) compared with subclinical cows ($31.5\% \pm 1.4$), with a tendency for higher numbers of monocyte-derived macrophages compared with the control cows ($P < 0.10$) (Fig. 6). Parturition did not affect the percentage of CD14⁺ cells in 8 d control PBMC. There was no overall effect of infection group or

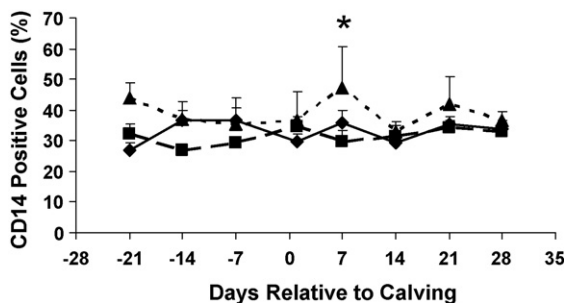


Fig. 6. Percentage of positive CD14 cells from 8 d control PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. Clinical cows expressed a greater percentage compared with subclinical cows ($P < 0.05$). Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

parturition on CD14⁺ cells from 8 d infected PBMCs (data not shown).

3.3. CD5 expression on mononuclear cell populations after fresh isolation or 8 d of culture

Overall, in freshly isolated PBMCs, clinical cows expressed lower ($P < 0.01$) percentages of CD4⁺CD5^{bright} T-cells and CD8⁺CD5^{bright} T-cells compared with the control cows and tended to have lower expression of these subpopulations than the subclinical cows ($P < 0.10$) (Fig. 7A and B). There was no overall effect of periparturient period. However, there was a significant increase ($P < 0.01$) in expression of CD4⁺CD5^{bright} T-cells for control cows between -14 d (46.82%) and the day of calving (79.2%). This same pattern was observed for CD8⁺CD5^{bright} T-cells. Control cows also expressed a higher ($P < 0.05$) percentage of $\gamma\delta$ TCR⁺CD5^{bright} T-cells in fresh PBMCs compared with the subclinically infected cows (Fig. 7C). Although parturition did not affect the percentage of $\gamma\delta$ TCR⁺CD5^{bright} T-cells, infected cows did have decreased expression in the postpartum period from +1 d through +21 d. Parturition did not affect the percentage of B-cellCD5^{bright} cells, but there was a strong trend towards lower expression for both subclinical ($P < 0.11$) and clinical ($P < 0.13$) cows compared with control cows (Fig. 7D).

In contrast, clinical cows expressed a greater percentage of CD4⁺CD5^{dim} cells in freshly isolated PBMCs compared with control ($21.6\% \pm 4.8$ vs. $40.44\% \pm 5.5$; $P < 0.05$). There was no overall effect of parturition, but the percentage of cells from subclinical cows increased from -7 d to +21 d ($P < 0.05$) and decreased in control cows from -14 d to +1 d ($P < 0.05$) (Fig. 8A). The percentage of CD8⁺CD5^{dim} cells was greater in clinically infected cows ($P < 0.01$) and tended to be greater in subclinically infected cows ($P < 0.06$) compared with the control cows (Fig. 8B). For clinical cows, the percentage rapidly increased from -7 d to +14 d ($P < 0.01$). During the postpartum period, subclinical and clinical cows expressed a greater percentage of $\gamma\delta$ TCR⁺CD5^{dim} compared with the control cows ($P < 0.05$) (Fig. 8C). Overall, clinical cows expressed a greater percentage of B-cellCD5^{dim} cells compared with the control cows ($P < 0.05$) (Fig. 8D).

There was no overall effect of infection or parturition on CD4⁺CD5^{bright} T-cells in 8 d control (Fig. 9A) or infected (Fig. 10A) PBMCs, although there was an interaction of infection group and parturition ($P < 0.09$). There was an overall effect of parturition

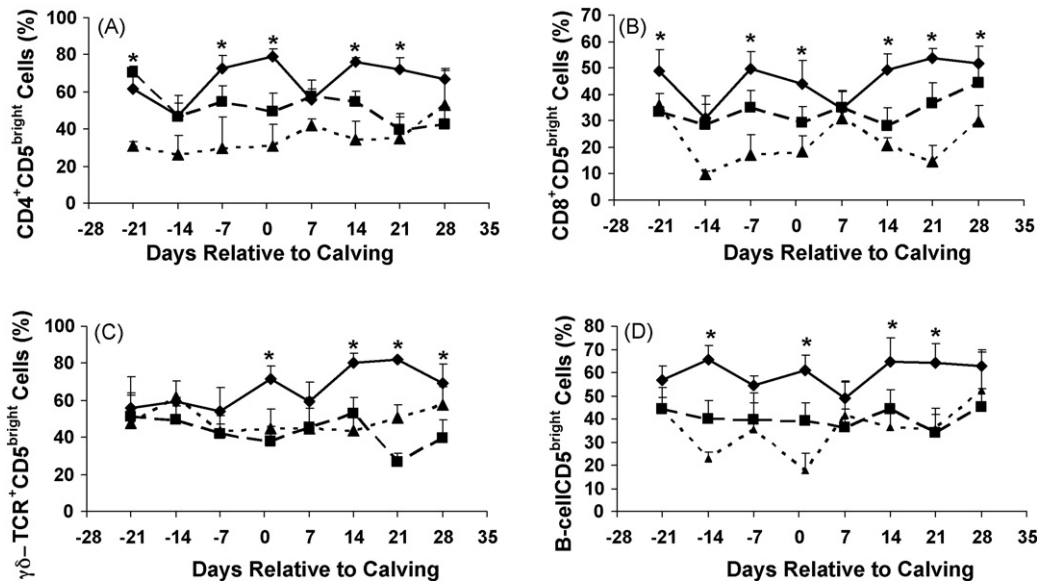


Fig. 7. Percentage of CD5^{bright} T-cells from fresh PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) CD4⁺ T-cells. Clinical cows expressed a smaller percentage compared with control cows ($P < 0.01$). (B) CD8⁺ T-cells. Clinical cows expressed a smaller percentage compared with control cows ($P < 0.05$). There was a tendency for a parturition effect ($P < 0.08$). (C) $\gamma\delta$ T-cells. Subclinical cows expressed a lower percentage than the control cows ($P < 0.05$). (D) B-cells. Data are least square means \pm S.E.M. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

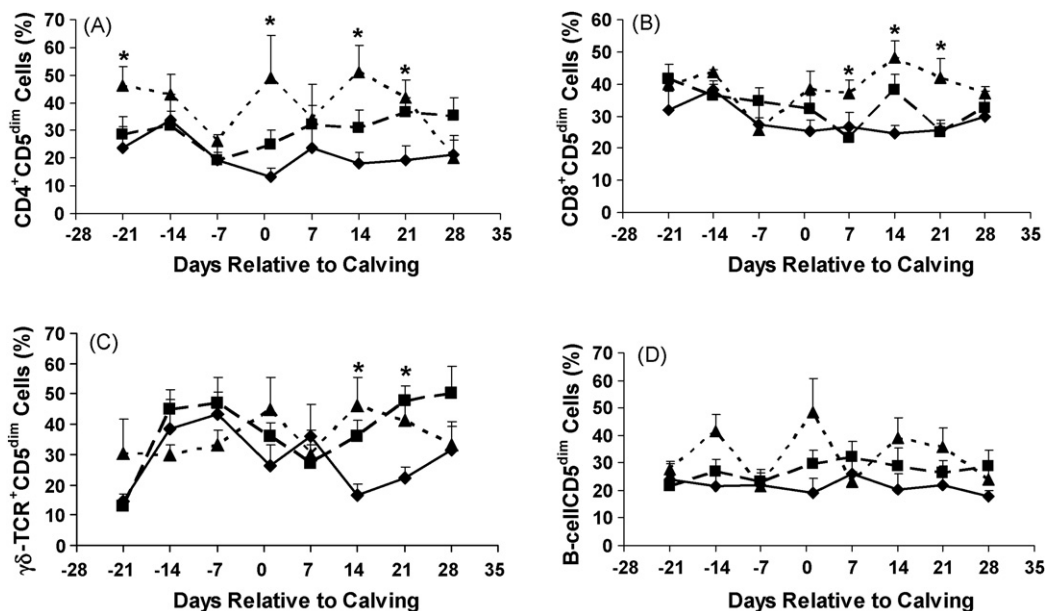


Fig. 8. Percentage of CD5^{dim} T-cells from fresh PBMCs from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) CD4⁺ T-cells. Clinical cows expressed a greater percentage compared with control cows ($P < 0.05$). (B) CD8⁺ T-cells. Clinical cows expressed a greater percentage compared with controls ($P < 0.01$) and subclinical ($P < 0.06$) cows. (C) $\gamma\delta$ T-cells. During postpartum period, subclinical and clinical cows had greater percentage compared with controls ($P < 0.05$). (D) B-cells. Clinical cows had greater percentage compared with controls ($P < 0.05$). Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

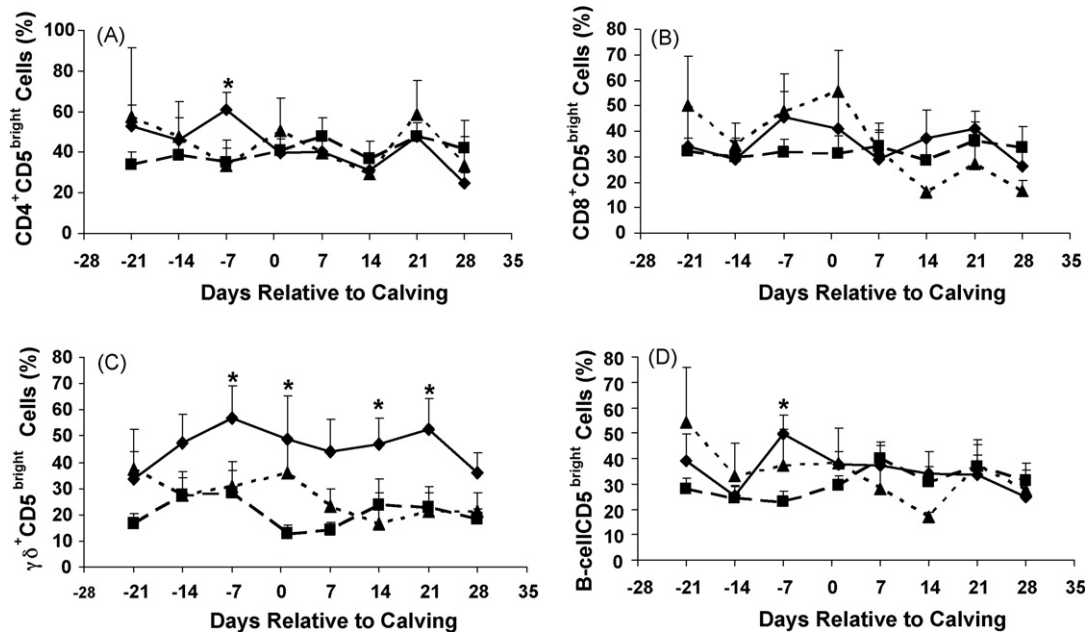


Fig. 9. Percentage of CD5^{bright} cells from 8 d PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) CD4⁺CD5^{bright} cells. (B) CD8⁺CD5^{bright} cells. There was an effect of infection group ($P < 0.01$). (C) $\gamma\delta$ ⁺CD5^{bright} cells. (D) B-cellsCD5^{bright} cells. Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

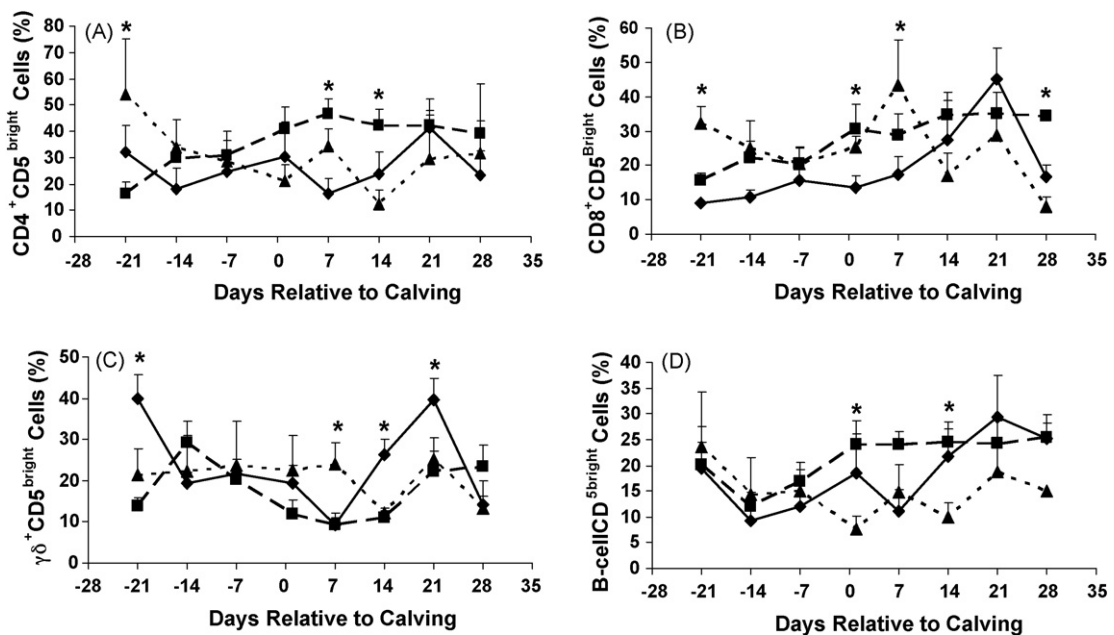


Fig. 10. Percentage of CD5^{bright} cells from 8 d infected PBMCs isolated from control (◆), subclinical (■), and clinical (▲) periparturient dairy cows. (A) CD4⁺CD5^{bright} cells. (B) CD8⁺CD5^{bright} cells. There was an effect of parturition ($P < 0.003$) and an interaction of infection and parturition ($P < 0.05$). (C) $\gamma\delta$ ⁺CD5^{bright} cells. There was an effect of parturition ($P < 0.01$) and an interaction of infection and parturition ($P < 0.004$). (D) B-cellsCD5^{bright} cells. Data are means \pm S.E. Significant differences between infection groups on a given day are represented by asterisks ($P < 0.05$).

($P < 0.07$) in 8 d control PBMCs (Fig. 9B) with overall percentages of CD8⁺CD5^{bright} T-cells decreasing from –21 d to +28 d ($P < 0.05$). In 8 d infected PBMCs, there was an overall effect of parturition ($P < 0.01$) and an interaction of infection group and parturition ($P < 0.01$). In the control cows, the percentage of $\gamma\delta$ TCR⁺CD5^{bright} T-cells from 8 d infected PBMCs, declined from $39.8\% \pm 5.8$ at –21 d to $9.2\% \pm 2.9$ by +7 d ($P < 0.01$) (Fig. 10C). The percentage then rapidly increased to +21 d. An effect of parturition was observed in 8 d infected PBMC as the percentage of B-cellCD5^{bright} cells increased in the prepartum period from –14 d to +1 d ($P < 0.01$) for subclinically infected cows, whereas an increase in B-cellCD5^{bright} cells was also observed from +7 d to +21 d postpartum in control cows ($P < 0.05$) (Fig. 10D).

4. Discussion

Infections caused by MAP have an overwhelming impact on the health of the dairy cow and ultimately on the dairy industry. Limited research has focused on changes in populations of peripheral blood mononuclear cells in dairy cows naturally infected with MAP during the periparturient period. The populations of B and T lymphocytes subsets in the peripheral blood have a direct impact on the ability of the animal to respond to new and existing infections.

In mycobacterial infections, CD4⁺ T-cells are recognized as the primary producer of IFN- γ , a key proinflammatory cytokine required for activation of macrophages and subsequent clearance of infection (Stuehr and Marletta, 1987; Flynn et al., 1993). In the current study, the number of CD4⁺ T-cells in freshly isolated PBMCs averaged $21.6\% \pm 2.1$, $24.3\% \pm 1.3$, and $25.7\% \pm 2.4$ for control, subclinical, and clinical cows, respectively, with no differences noted between the infection groups. These percentages are consistent with the 25–35% reported in the literature for healthy adult dairy cattle. The lack of effect of MAP infection on the percentages is in agreement with a previous study that did not observe differences in CD4⁺ T-cell percentages in PBMCs isolated from MAP-infected periparturient dairy cows (Harp et al., 2004). Interestingly, stimulation of 8 d PBMCs with MAP resulted in an increase in the percentage of CD4⁺ T-cells during the prepartum period for subclinical cows with a decrease noted for clinically infected cows. These data would suggest that CD4⁺ T-cells from subclinically cows are able to proliferate in response to antigen during periods of physiologic stress, whereas clinically cows may be compromised in their ability to respond.

CD8⁺, or cytotoxic T-cells are important for their ability to recognize viral and bacterial antigens and to target T-cells displaying these antigens for apoptosis (Kagi et al., 1994). Similar to CD4⁺ T-cells, CD8⁺ T-cells also secrete pro-inflammatory cytokines, IFN- γ and TNF- α , making them equally important in controlling infection (Fong and Mosmann, 1990; Berg et al., 2002). In the current study, the percentage of CD8⁺ T-cells in freshly isolated PBMCs ranged from 10% to 22% which is equivalent to published values for adult dairy cows (Harp et al., 1991; Meglia et al., 2005). Although it has been suggested that these cells are not influenced by parturition (Harp et al., 1991, 2004), we observed in freshly isolated PBMCs from subclinically infected cows a two-fold increase in the percentage of CD8⁺ T-cells during the postpartum period. Koets et al. (2002) reported no differences in percentages of CD8⁺ T-cells between healthy cows and cows with subclinical and clinical paratuberculosis. One explanation for the increase in CD8⁺ cell number in the subclinically infected cows may be that this T-cell population is highly activated as a result of the cytokine micro-environment associated with the initial stages of MAP-infection and that this response provides an early defense system against the invading bacteria (Lertmoungkolchai et al., 2001; Yajima et al., 2002).

The CD4 to CD8 T-cell ratio is often used as an indicator of immune status. For example, in HIV-infected populations, a diminished CD4:CD8 ratio may be used to accurately predict the occurrence of an AIDS-related complication (Pirzada et al., 2006). When compared with subclinically infected cows, clinical cows have diminished CD4:CD8 ratios in the peripheral blood, with little variation in the percentage of CD8⁺ T-cells (Chiodini and Davis, 1992; Koets et al., 2002). In our study, in the fresh PBMCs, the periparturient period triggered differences in the peripheral blood CD4:CD8 ratios between cows in different stages of infection. Subclinically infected cows had a lower CD4:CD8 ratio, whereas the clinically infected cows had higher ratios during the immediate postpartum period. The CD4:CD8 ratio does not seem to be influenced by parturition (Van Kampen and Mallard, 1997; Kimura et al., 1999). Our results suggest there was an interaction between the effects of MAP infection and the dynamic events occurring during the periparturient period.

$\gamma\delta$ T-cells seem to initiate immune responses and may regulate host inflammatory response to infection although their specific roles in host immunity are still relatively undefined. The percentage of $\gamma\delta$ T-cells is greatest in the calf (40%) and gradually declines to approximately 5% of adult PBMCs (Hein and MacKay,

1991). In freshly isolated PBMCs, clinical cows had a much lower percentage of $\gamma\delta$ T-cells compared with subclinical and control cows. This response is in agreement with a study reporting that the population of $\gamma\delta$ T-cells from freshly isolated PBMCs was much greater in control cows (21.8%) compared with MAP-infected clinical cows (7.3%) (Koets et al., 2002). The exact function of $\gamma\delta$ T cells remains to be determined, although there is evidence to suggest that these cells play a significant role in the innate immune response to initial mycobacterial infection. Early infection with *M. tuberculosis* resulted in increased numbers of activated $\gamma\delta$ T cells that were capable of producing the proinflammatory cytokine, IFN- γ (Tsukaguchi et al., 1995). Furthermore, stimulation of bovine $\gamma\delta$ T cells with mycobacterial products resulted in expansion and production of IFN- γ (Vesosky et al., 2004). The lower percentage of $\gamma\delta$ T cells observed in the clinical cows in our study is most likely an attempt to limit the severe inflammation and tissue damage caused by an abundance of proinflammatory cytokines in the subclinical stage of Johne's disease.

In the current study, the percentage of B-cells across infection groups in fresh PBMCs ranged from 28% to 35%. Neither infection status of the cows nor parturition had an effect on the overall percentages of B-cells in fresh or 8 d culture. In the advanced stages of Johne's disease, antibody production by B-cells does little to protect the host from the progressive MAP-infection. Waters et al. (1999) reported that population percentages of B-cells in peripheral blood were similar between subclinical and control cows but were greater in clinical cows. In our study, the increase in percentage of B cells from -14 d to +7 d in fresh PBMCs for clinically infected cows is an indication of activated humoral response around the time of calving. Clinically infected cows had a lower percentage of B cells in fresh PBMCs prepartum and at calving compared with subclinical and control cows. The fact that this value returned to a normal level by +7 d may be an indication of activated humoral response in clinical cows during the postpartum period. This may also be an indication of the shift to Th2 immunity that has been documented in dairy cows at parturition (Shafer-Weaver et al., 1999).

CD5 is expressed on all bovine T-cells and a subset of B-cells (Depelchin et al., 1989; Naessens and Williams, 1992). In T-cells, CD5 is a regulating component of TCR-signal transduction during the double-positive stage of lineage commitment (Tarakhovsky et al., 1995). We have identified previously two subpopulations of CD5⁺ cells (CD5^{bright} and CD5^{dim}) in the bovine that are modified by paratuberculosis infection. Both CD5 bright and dim

populations were examined as a percentage of marker positive lymphocyte subsets. For CD4⁺, CD8⁺, and B-cell subsets isolated from fresh PBMCs, clinical cows expressed the lowest percentage of CD5^{bright} cells and the greatest percentage of CD5^{dim} cells compared with the control cows. A lower percentage of CD5^{bright} cells in the peripheral blood of clinical cows may be an indication that the activated lymphocytes are being sequestered at the site of infection. During the clinical stage of disease there is a high bacterial load (Hostetter et al., 2005) and an increase in lymphocytes in the granulomas at the site of infection. It is significant that clinically infected cows expressed a greater percentage of CD5^{dim} B-cells (21.8% \pm 3.6 in control cows vs. 33.4% \pm 4.2 in clinical cows) because these cells are capable of producing the Th2 cytokine, IL-10 (Gieni et al., 1997). It has been identified that the IL-10 produced by CD5⁺ B-cells acts directly on macrophages to decrease IL-12 secretion (Sun et al., 2005). On the basis of our data, we hypothesize that the increased proliferation of CD5^{dim} B-cells by clinical Johne's cows directly inhibits cell-mediated immune responses by altering the cytokine microenvironment in favor of a Th2 response.

One objective of this study was to evaluate the naïve and recall responses of PBMCs to MAP-infection by isolating cells from healthy control cows and from cows that have been exposed to MAP by natural infection. We hypothesized that PBMCs isolated from subclinical and clinical cows and cultured for 7 d would proliferate in response to in vitro infection with live MAP at a greater proportion than the cells isolated from the control animals. However, of the cells examined, only monocyte-derived macrophages from clinically infected cows increased in response to stimulation compared with subclinical and control cows. The ability of lymphocytes to respond to MAP infection may be suppressed during the periparturient time period. Both the innate and adaptive immune responses are lowest during this time (Mallard et al., 1997). Furthermore, lymphocyte function is diminished at parturition (Kehrli et al., 1989a).

The results from this study contribute to the limited available information that focuses on the impact periparturient immunosuppression may have on the ability of the host to respond to progressing MAP infection. Stress induced by parturition has a major impact on the number of T- and B-cells in the peripheral blood. Our data clearly supports an interaction of parturition and infection status on these lymphocyte subsets. This interaction is best illustrated in freshly isolated PBMCs by the upregulation of CD4⁺ in clinical cows and an increase in CD8⁺ in subclinical cows at parturition. In these animals, these lymphocyte subsets

are responding in the host in an attempt to contain and limit the MAP infection. Manipulating these changes in lymphocyte populations may be one mechanism to assist the infected dairy cow in attempting to manage the highly stressful periparturient period.

5. Conclusions

Results of this study indicate that in dairy cows the percentages of both lymphocyte subsets and mononuclear cells are modulated by natural infection with MAP and by the periparturient period. In addition, these factors are capable of influencing the expression of the activation marker, CD5, on T cell subpopulations and B cells. The data presented are important because they highlight changes in the immune response of infected cattle at parturition that may be an attempt to limit the progression of Johne's disease during the highly stressful time of parturition.

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